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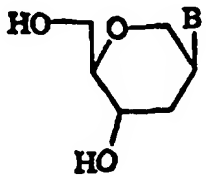
(57) Abstract

The invention relates to oligomers consisting completely or partially of 1,5-anhydrohexitol nucleoside analogues represented by general formula (I), wherein B is a heterocyclic ring which is derived from a pyrimidine or purine base, such as cytosine, 5-methylcytosine, uracil and thymine, or deaza derivatives thereof, or adenine, guanine, 2,6-diaminopurine, hypoxanthine and xanthine, or deaza derivatives thereof.



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SEQUENCE-SPECIFIC BINDING OLIGOMERS FOR NUCLEIC ACIDS AND THEIR USE IN ANTISENSE STRATEGIES

The present invention relates to oligomers having nucleic acid binding properties, which oligomers completely or partially consist of 1,5-anhydrohexitol nucleoside analogues as monomeric units. The invention further relates to the use of the oligomers in antisense techniques and to a method of preparing the oligomers.

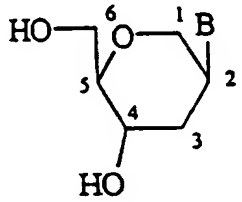
Antisense techniques are based on the principle that the function of a coding sense strand of a DNA or RNA molecule may be blocked by a complementary antisense strand. Antisense techniques may be used for various applications, such as diagnosis, therapy, DNA modification and isolation etc.. In these techniques, besides the stability of the antisense strand itself, the stability of the duplex or triplex formed by the sense and antisense strands as well as the binding affinity of the antisense strand for the sense strand are of importance. Likewise, the sensitivity of the oligomer, the duplex or the triplex for degrading enzymes, such as nucleases, is a factor relevant for the effectivity.

Oligonucleotides are oligomers in which the monomers are nucleotides. Nucleotides are phosphate esters of nucleosides, which are built of a purine or pyrimidine base and a sugar. The backbone of each nucleotide consists of alternating sugars and phosphate groups.

The stability and binding affinity of the nucleotides may for example be influenced by modification of the base. Research in that direction (1-5) showed that such modifications only lead to less stable duplexes. Alterations in the backbone or the incorporation of new structures therein did lead to an increased nuclease stability but had only an adverse effect on their binding affinity for complementary strands. Modification of the sugars led to a merely limited increase in the affinity for the target molecule (6-8).

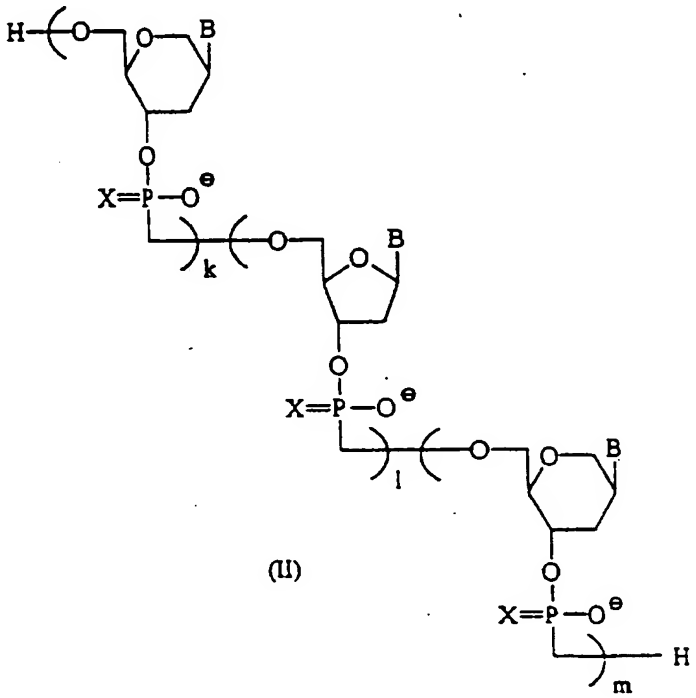
It is the object of the present invention to provide new oligomers, which have an improved stability and binding affinity as compared to the known oligomers.

It has now been found that oligomers, consisting completely or partially of 1,5-anhydro-2,3-dideoxy-D-arabino-hexitol nucleoside analogues, wherein the hexitol is coupled via its 2-position to the heterocyclic ring of a pyrimidine or purine base, are capable of binding to naturally occurring oligonucleotides. The monomers of which the oligomers are at least partially composed are presented by the formula I:



(I)

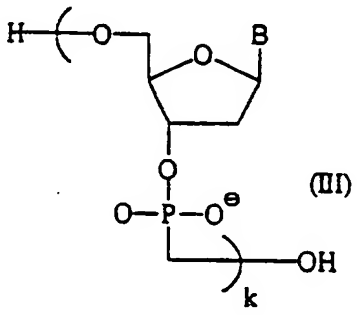
wherein B is a heterocyclic ring which is derived from pyrimidine or purine base. The monomers are connected to each other through a phosphordiester bridge with formula II representing the structure of these oligomers,



(II)

wherein B is a heterocyclic ring which is derived from a pyrimidine or purine base and, wherein l is an integer from 0 to 15, k and m each are integers from 1 to 15, but if k > 1, then m may be 0 and if m > 1, k may be 0; and, wherein X represents oxygen or sulfur. All possible salts of the compound of formula II are included in the invention. The monomers of formula I are the subject of European patent application No. 92201803.1. The oligomers of formula II are novel compounds. They display a certain similarity with oligonucleotides consisting of the naturally occurring 2'-deoxynucleosides, but the sugars of the monomers are enlarged because a methylene group is incorporated in between the ring oxide and the carbon, which is coupled to the base.

According to the invention it has been found that the oligomers of formula II and their salts exhibit sequence specific binding to natural oligonucleotides represented by formula III



(III)

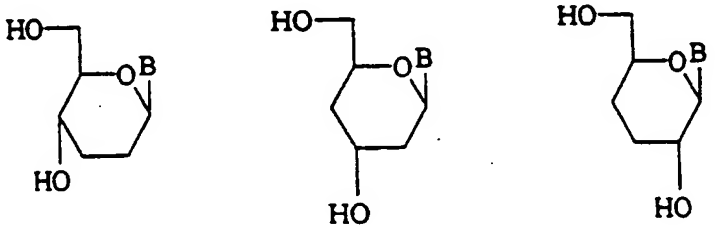
wherein k is an integer and wherein B has the same designation as in formula's I and II. A new class of hybridons or sequence-specific binding polymers has therefore been found.

The fact that oligomers according to the invention, consisting at least partially of pyranose nucleosides, have a high binding affinity is very surprising. The study of oligonucleotides built up from monomeric pyranose nucleotides has been undertaken over the past years inter alia by the group of A. Eschenmoser et al.. Eschenmoser investigated nature's selection of furanoses as sugar building blocks for nucleic acids (9). However, he did

not indicate the requirements which a suitable antisense molecule should meet to accomplish a good binding to naturally occurring furanose-DNA.

The present inventors however investigated which pyranose-like oligonucleotide would be able to form stable duplexes with natural furanose-DNA (10, 11). Theoretically, a pyranose oligonucleotide has a free energy advantage over a furanose oligomer because of less entropy changes during duplex formation.

However, the pyranose-like oligonucleotides studied by the present inventors before were not able or not sufficiently able to bind to complementary strands of natural furanose-DNA. These pyranose-like oligonucleotides consisted of 2,3-dideoxy-B-D-erythro-hexopyranosyl nucleosides (formula V), 2,4-dideoxy-ß-D-erythro-hexopyranosyl nucleosides (formula VI) and/or 3,4-dideoxy-ß-D-erythro-hexapyranosyl nucleosides (formula VII), respectively.



V VI VII

The fact that sequence-specific binding is found for the oligomers of formula II, comprising pyranoses as sugar building blocks is therefore even more surprising. Enlarging the furan ring of furanose compounds to a pyran ring did not yield oligomers capable of binding natural oligonucleotides. Thus, the effect of enlarging the pento-furanosyl ring to a 1,5-anhydrohexitol ring could not be anticipated.

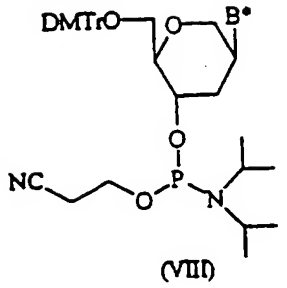
The compounds according to the invention are therefore oligomers of nucleoside analogues wherein a 1,5-anhydro-2,3-dideoxy-D-hexitol is coupled via its 2-position according to an arabino-configuration to the heterocyclic ring of a pyrimidine or purine base.

The oligomers consist of the above nucleoside analogues connected to each other as phosphate diesters or thiophosphate diesters. The oligomers can be represented by the formula II wherein k, l, m, B and X have the above stated designations. The oligomers can be exclusively composed of the hexitol nucleoside analogues of the formula I (with l in formula II equalling zero) or can have natural 2'-deoxynucleosides interspersed or at the end of the molecule (with l in formula II equalling one or greater). The hexitol has the (D)-configuration and the stereochemistry of the substituents is according to an arabino configuration.

When group B is derived from a pyrimidine base it can be either cytosine, 5-methyl cytosine, uracil or thymine. When B is derived from a purine base it can be an adenine, guanine, 2,6-diaminopurine, hypoxanthine or xanthine ring, or a deaza derivative of one to these.

The nucleoside analogues, monomer components of the present invention, can be prepared in different ways and one of the preparation methods is subject of the European patent application no. 92.201803.1. These syntheses haven been described likewise in Verheggen et al. (12). Assembly of the monomers into an oligomer follows the classical schemes and can be done either by standard phosphoramidite chemistry (compare ref. 13) or by H-phosphorate chemistry (compare ref. 14). All procedures are conveniently carried out on an automated DNA synthesizer as for standard oligonucleotide synthesis. For these standard conditions reference is made to Methods in Molecular Biology (15).

The preferred method is the phosphoramidite method making use of the phosphoramidites of the hexitol nucleoside analogues as the incoming building blocks for assembly in the "6'-direction". The phosphoramidites are represented by formula VIII wherein B^* is a protected base moiety suitable for oligonucleotide synthesis (e.g. thymine, N^4-benzoyl-cytosine, N^6-benzoyladenine en N^2-isobutyrylguanine, represented by the formula's IX, X, XI and XII, respectively).



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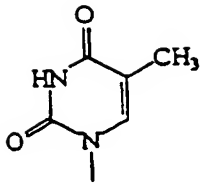

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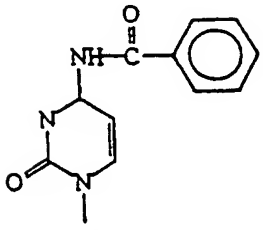

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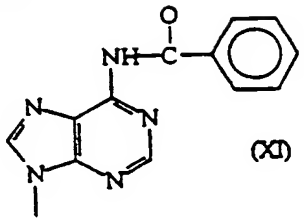

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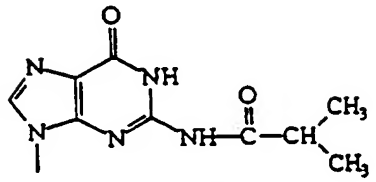

(XII)

The products of formula VIII can be prepared according to standard procedures. Protection of the base moieties of cytosine, adenine or guanine is accomplished following a transient protection strategy for the hydroxyl moieties of the compounds of formula I (16). Preferably, however, the base protection is carried out by acylation of the 4,6-benzylidene protected nucleoside analogues 1a-d, which are intermediates in the synthesis of the monomers of the above stated formula I.

Following acylation of the exocyclic amino functionality, the benzylidene moiety is removed with 80% acetic acid to obtain 3a-d. To obtain compound 3c the p-nitro-phenylethyl group can be removed with DBU.

The primary hydroxyl function of the 1,5-anhydro-hexitol analogues 3a-d can be protected with a dimethoxy-trityl group to yield 4a-d. Conversion to the phosphor-amidite building blocks 5a-d suitable for incorporation into an oligonucleotide chain can be accomplished with 2-cyano-ethyl N,N-diisopropylchlorophosphoramidite. Supports containing a 1,5-anhydrohexitol analogue can be prepared by succinylation of the compounds 4a-d yielding 6a-d, which can be coupled to the amino function of either long chain

alkylamino controlled pore glass (CCAA-CPG) or a suitable amino functionalized polystyrene (e.g. Tentagel®-RAPP Polymere) making use of a carbodiimide, and yielding **7a-d** (for functionalization of supports viz. ref. 17)

After assembly, the obtained oligonucleotides are cleaved from the support and deprotected by ammonia treatment for 16 hours at 55°C. Purification of the obtained oligomers of the above stated formula II can be accomplished in several ways (18). The preferred method is purification by anion-exchange FPLC at a basic pH of 12 to disrupt all possible secondary structures (10). Desalting can be performed by simple gel filtration techniques followed by lyophilization. All acceptable salts can be prepared in conventional manner.

1a-d
2 a-d
3 a-d : R1 = H
4 a-d : R1 = DMTr
5 a-d : R2 = P(N(iPr)2)OCH2CH2CN
6 a-b : R2 = C(=O)CH2CH2COOH
7a-b : R2 = C(=O)CH2CONH----CPG
a : B = thymin-1-yl
b : B = adenin-9-yl
c : B = N2-isobutyryl-O6-(2-(p-nitrophenyl)ethyl)guanin-9-yl
d : B = cytosine-1-yl
a: B* = thymin-1-yl = B
b: B* = N6-benzoyladenin-9-yl
c: B* = N2-isobutyrylguanin-9-yl
d: B* = N4-benzoylcytosin-1-yl
CPG = controlled pore glass (solid support)
(i) 80% HOAc; (ii) dimethoxytrityl chloride, pyridine; (iii) N,N-diisopropylethylamine, 2-cyano-N,N-diisopropylchlorophosphoramidite, CH2Cl2; (iv) DMAP, succinic anhydride, pyridine; (v) pre-activated LCAA-CPG, DMAP, Et3N, 1-(3-diethylaminopropyl)-3-ethylcarbodiimide.HCl, pyridine.

As stated above, the oligomers display sequence-specific binding to natural oligonucleotides. They show stronger binding to a complementary natural oligodeoxynucleotide than the unmodified sequence and they are endowed with much higher biochemical stability. In this manner they can advantageously be used for antisense strategies which comprise diagnosis, hybridization, isolation of nucleic acids, site-specific DNA modification and therapeutics and all anti-sense strategies currently being pursued with natural oligodeoxynucleotides.

EXAMPLES

The compounds according to the invention as well as their chemical synthesis and the preparation of starting materials are further illustrated in the following examples, which are not however intended to limit the invention.
The following abbreviations are being used:
FABMS = fast atom bombardment mass spectrometry
Thgly = thioglycerol
NBA = nitrobenzylalcohol

Synthesis of the 1,5-anhydro-2,3-dideoxy-2-substituted-D-arabino-hexitol nucleoside analogues and of their 4,6-O-benzylidene protected derivatives has been described by Verheggen et al. (12).

EXAMPLE 1

Base-protected nucleoside analogues

1.1. 1,5-anhydro-2-(N^6-benzoyladenin-9-yl)-2,3-dideoxy-D-arabinohexitol (3b)

To a solution of 2.3 g (6.51 mmol) 1,5-anhydro-4,6-O-benzylidene-2-(adenin-9-yl)-2,3-dideoxy-D-arabino-hexitol in 20 ml of dry pyridine, 0.9 ml (7.8 mmol) of benzoylchloride was added at 0°C. After stirring for 4 hours at room temperature, the mixture was cooled on an ice bath and 2 ml of H_2O was added. After addition of 1.5 ml of a concentrated NH_3 solution (33% g/v) and further stirring for 45 min. at room temperature, the mixture was evaporated. The

residue was purified by column chromatography (CH_2Cl_2-MeOH, 98:2) yielding 1.92 g (4.19 mmol, 64% yield) of 1,5-anhydro-4,6-O-benzylidene-2-(N^6-benzoyladenin-9-yl)-2,3-dideoxy-D-arabinohexitol.

This was further treated with 100 ml of 80% acetic acid at 60°C for 5 hours to remove the benzylidene moiety. Evaporation, coevaporation with toluene and purification by column chromatography (CH_2Cl_2-MeOH, 95:5 tot 90:10) yielded 1.10 g (2.98 mmol, 71% yield) of the compound mentioned in the title of this example.

UV(MeOH) λ_{max}282nm(ϵ=20200)

FABMS (Thgly, NaOAc) m/e:392$(M+Na)^+$.240$(B+2H)^+$

^{1}H NMR (DMSO-d_6 δ 1.94(m, 1H, H-3'ax), 2.32 (m, 1H. H-3'eq), 3.21 (m, 1H, H-5'), 3.42-3.76 (m, 3H, H-4', H-6', H-6"), 3.90 (dd, ^{2}J=13Hz, 1H, H-1'ax), 4.27 (dd, ^{2}J=12.2Hz, 1H, H-1'eq), 4.67 (t, J=5.7Hz, 1H, 6'=OH), 4.88-5.00 (m, 2H, H-2', 4'-OH), 7.47-7.68 (m, 3H, aromatic H), 8.00-8.07 (m, 2H aromatic H) 8.60 (s, 1H), 8.73 (s, 1H) (H-2, H-8) ppm.

^{13}C NMR (DMSO-d_6) δ 35.8 (C-3'), 50.7 (C-2'), 60.5, 60.7 (C-4', C-6'), 67.9 (C-1'), 83.1 (C-5'), 125.1 (C-5), 128.5 (Co, Cm), 132.5 (Cp), 133.6 (Cx), 143.5 (C-8), 150.3 (C-4), 151.4 (C-2), 152.4 (C-6) ppm.

1.2. 1,5-Anhydro-2,3-dideoxy-2-(N^2-isobutyrylguanin-9-yl-D-arabinohexitol (3c)

Alkylation of N^2-isobutyryl-O^6-[2-(p-nitrophenyl) ethyl]guanine (1.85 g, 7.5 mmol) with 1,5-anhydro-4,6-O-benzylidene 3-deoxy-D-glucitol (1.18 g, 5 mmol) yielded 1.35 g of crude 1,5-anhydro-4,6-O-benzylidene-2,3-dideoxy-2-(N^2-isobutyryl-guanin-9-yl)-D-arabinohexitol after removal of the p-nitrophenyl-ethylgroup with 1.5 ml (10 mmol) of DBU in anhydrous pyridine for 16 hours and purification by flash column chromatography (CH_2Cl_2-MeOH, 99:1 to 97:3).

Hydrolysis of the benzylidene moiety with 100 ml of 80% HOAc (5 hours at 60°C) gave the desired compound 3c (610 mg, 1.74 mmol, 34% overall yield) after column chromatography (CH_2Cl_2-MeOH, 90:10).

UV(MeOH) λ_{max}273nm

FABMS (Thgly, NaOAc) m/e:352(M+H)$^+$
^{1}H NMR δ 1.11 (d, J = 6.7 Hz, 6H, CH_3), 1.93 (m, 1H, H-3'ax), 2.11-2.38 (m, 1H. H-3'eq), 2.80 (q, 1H, CHMe-2), 3.25 (m, 1H, H-5'), 3.42-3.78 (m, 3H, H-4', H-6', H-6"), 3.89 (dd, ^{2}J=13Hz, 1H, H-1'), 4.21 (dd, ^{2}J=13Hz, 1H, H-1"), 4.69
^{13}C NMR δ 19.4 (CH_3), 34.5 ($CHMe_2$), 35.8, (C-3'), 50.5 (C-2'), 60.5, 60.7 (C-4', C-6'), 67.9 (C-1'), 83.1 (C-5'), 116.7 (C-5), 141.7 (C-8), 152.0 (C-4), 153.0 (C-2), 159.8 (C-6), 175.2 (C=O) ppm.

EXAMPLE 2

Dimethoxytritylation van de nucleoside analogues

2.1. 1,5-Anhydro-6-O-dimethoxytrityl-2-(thymin-1-yl)-2,3-dideoxy-D-arabinohexitol (4a)

1,5-Anhydro-2-(thymin-2-yl)-2,3-dideoxy-D-arabino-hexitol (3a) (330 mg, 1.29 mmol) was dissolved in 20 ml of anhydrous pyridine, and 480 mg (1.42 mmol) of dimethoxytrityl chloride was added. The mixture was stirred overnight at room temperature, diluted with 100 ml of CH_2Cl_2 and washed twice with 100 ml of saturated $NaHCO_3$ solution. The organic layer was dried, evaporated and coevaporated with toluene. The resulting residue was purified by column chromatography (with a gradient of 0 to 3% MeOH in $CHCl_3$ containing 1% triethylamine) to yield 373 mg (0.67 mmol, 52%) of the title compound as a foam.
FABMS (Thgly, NaOAc) m/e:581(M+Na)$^+$.127 (B+2H)$^+$
^{1}H NMR ($CDCl_3$): δ 1.60-2.50 (m, 2H, H-3', H-3"), 1.91 (s, 3H, CH_3), 3.12-3.62 (m, 2H, H-5', H-4'), 3.77 (s, 6H, 2x OCH_3), 3.65-4.17 (m, 4H, H-6', H-6", H-1', H-1"), 4.53 (s, 1H, H-2'), 4.88 (d, 1H, J=5.1, Hz 4'-OH), 6.81 (d, J=8.7, 4H, aromatic H), 7.09-7.53 (m, 9H, aromatic H), 8.09 (s, 1H, H-6), 9.10 (br s, 1H, NH) ppm
^{13}C NMR ($CDCl_3$) δ 12.5 (CH_3), 35.5 (C-3'), 50.7 (C-2'), 54.9 (OCH_3), 62.4, 63.1 (C-4', C-6'), 68.2 (C-1'), 81.1 (C-5'), 86.0 (Ph_3C) 110.0 (C-5), 138.4 (C-6), 151.0 (C-2), 163.8 (C-4), 112.9, 126.6, 127.5, 127.8, 129.7, 135.6, 144.6, 158.3 (aromatic C) ppm.

2.2. 1,5-Anhydro-6-O-dimethoxytrityl-2-(N^6-benzoyladenin-9-yl)-2,3-dideoxy-D-arabinohexitol (4b)

A solution of 370 mg (1 mmol) of the nucleoside 3b and 400 mg (1.2 mmol) of dimethoxytritylchloride in 25 ml of pyridine dry was stirred at room temperature for 16 hours. The mixture was diluted with 100 ml of CH_2Cl_2 and washed twice with 100 ml of saturated $NaHCO_3$ solution. The organic layer was dried, evaporated and coevaporated with toluene. The residue was purified by column chromatography (0 to 3% of MeOH in CH_2Cl_2 with 0.2% pyridine) to obtain 400 mg (0.6 mmol, 63% yield) of compound 4b as a foam. FABMS (Thgly, NaOAc) m/c: 694 $(m+Na)^+$, 240 $(B+2H)^+$.

2.3. 1,5-Anhydro-6-O-dimethoxytrityl-2-(N^2-isobutyrylguanin-9-yl)-2,3-dideoxy-D-arabinohexitol (4c)

A solution of 580 mg (1.65 mmol) of the nucleoside 3c and 670 mg (2.0 mmol) of dimethoxytritylchloride in 25 ml of dry pyridine was stirred at room temperature for 16 hours. The mixture was diluted with 100 ml of CH_2Cl_2 and washed twice with 100 ml of saturated $NaHCO_3$ solution. The organic layer was dried, evaporated and coevaporated with toluene. The residue was purified by column chromatography with a gradient of 0 to 3% MeOH in CH_2Cl_2 containing 0.2% pyridine to obtain 770 mg (1.18 mmol, 71% yield) of compound 4c as a foam. FABMS (NBA) m/e: 654 $(M+H)^+$

2.4. Preparation of the amidite building blocks (5a-c)

A mixture of the 6'-O-protected nucleoside (0.5 mmol), 3 equivalents of dry N,N-diisopropyl-ethylamine and 1.5 equivalents of 2-cyanoethyl-N,N-diisopropylchloro-phosphoramidite in 2.5 ml of dry CH_2Cl_2 was stirred at room temperature for 3 hours. After addition of 0.5 ml of EtOH and further stirring for 25 min, the mixture was washed with 5% $NaHCO_3$-solution (15 ml) and saturated NaCl solution, dried and evaporated. Flash column chromatography with Et_3N afforded the amidite as a white foam which was dissolved in a small amount of dry CH_2Cl_2 and added dropwise to 100 ml of

cold (-50°C) n-hexane. The precipitate was isolated, washed with n-hexane, dried and used as such for DNA synthesis.

The following table gives the eluting solvent and yield after precipitation for the different amidites:

compound	solvent	solvent ratio	yield	FABMS(NBA) m/e
5a	n-hexane/ethyl acetate/ triethylamine	23:75:2	62%	759 $(M+H)^+$
5b	n-hexane/ethyl acetate/ triethylamine	50:48:2	65%	872 $(M+H)^+$
5c	n-hexane/acetone/ triethylamine	55:43:2	56%	854 $(M+H)^+$

EXAMPLE 3

Succinylation of the 6-O-protected nucleoside analogues

3.1. 1,5-Anhydro-6-O-dimethoxytrityl-4-O-succinyl-2-(thymin-1-yl)-2,3-dideoxy-D-arabinohexitol(6a)

A mixture of 80 mg (0.14 mmol) 4a, 9 mg (0.07 mmol) of DMAP and 43 mg (0.14 mmol) of succinic anhydride in 5 ml of anhydrous pyridine was stirred at room temperature for 24 hours. As the reaction was incomplete an additional amount of 43 mg (0.43 mmol) was added and the mixture was stirred for another 24 hours. The solution was evaporated and coevaporated with toluene. The residue was dissolved in CH_2Cl_2, the organic layer washed with saturated NaCl solution and water, dried and evaporated to give 78 mg (0.12 mmol, 86% yield) of 6a as a white foam.

3.2. 1,5-Anhydro-6-O-dimethoxytrityl-4-O-succinyl-2-(N^6-benzoyladenin-9-yl)-2,3-dideoxy-D-arabinohexitol (6b)

The same procedure as described for 6a was used for the synthesis of 6b. An amount of 260 mg (0.39 mmol) of 4b yielded 256 mg (0.33 mmol, 85% yield) of the captioned compound as a foam.

EXAMPLE 4

Production of oligonucleotides

4.1. Preparation of solid support

A mixture of 80 μmol of the succinates (6a, b), 400 mg of pre-activated LCAA-CPG (17), 5 mg (40 mmol) of DMAP, 35 μl of Et_3N and 153 mg of (800 μmol) 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide.HCl in 4 ml of anhydrous pyridine was first sonicated for 5 min and then shaken at room temperature for 16 hours. After shaking, the CPG solid support was filtered off and washed successively with pyridine, methanol and CH_2Cl_2 followed by drying under vacuum. The unreacted sites on the surface of the support were capped using 1.5 ml of 1-methylimidazole in THF (Applied Biosystems) and 1.5 ml of acetic anhydride-lutidine-THF 1:1:8 (Applied Biosystems). After shaking for 4 hours at room temperature, the solid support was filtered off, washed with CH_2Cl_2 and dried under vacuum. Colorimetric dimethoxytrityl analysis indicated a loading of 18.5 μmol/g for 7a and 21.5 μmol/g for 7b.

4.2. DNA-synthesis

Oligonucleotide synthesis was performed on an ABI 381A DNA synthesizer (Applied Biosystems) using the phosphoramidite method (end dimethoxytrityl off). The obtained sequences were deprotected and cleaved from the solid support by treatment with concentrated ammonia (55°C, 16 hours). After purification on a NAP-10® column (Sephadex G25-DNA grade, Pharmacia), eluted with buffer A (see below), purification was done on a mono-Q® HR 10/10 anion exchange column (Pharmacia) with the following gradient system [A= 10 mM NaOH, pH 12.0, 0.1 M NaCl; B= 10 mM NaOH, pH 12.0, 0.9 M NaCl; gradient used depended on the oligo; flow rate 2 ml/min]. The low pressure liquid chromatography system consisted of a Merck-Hitachi L6200 A Intelligent Pump, a Mono Q® HR 10/10 column (Pharmacia), an Uvicord SJI 2138 UV detector (Pharmacia-LKB) and a recorder. The product containing fraction was desalted on a NAP-10® column and lyophilized.

EXAMPLE 5

Melting temperatures

Oligomers were dissolved in the following buffer: 0.1 M NaCl, 0.02 M potassium phosphate pH=7.5, 0.1 mM EDTA. The concentration was determined by measuring the absorbance at 260 nm at 80°C and assuming the 1,5-anhydrohexitol nucleoside analogues to have the same extinction coefficients in the denatured state as the natural nucleosides.

For the adenine monomers ϵ = 15000

For the thymine monomers ϵ = 8500

For the guanine monomers ϵ = 12500

For the cytosine monomers ϵ = 7500

The concentration in all experiments was approximately 4 μM of each strand. Melting curves were determined with a Uvikon 940 Spectrophotometer. Cuvettes were thermostated with water circulating through the cuvette holder and the temperature of the solution was measured with a thermistor directly immersed in the cuvette. Temperature control and data acquisition were done automatically with an IBM/Pc AT compatible computer. The samples were heated and cooled at a rate of 0.2°C/min and no difference could be observed between heating and cooling melting curves, Melting curves were evaluated by taking the first derivative of the absorbance versus temperature curve. Examples of the synthesized oligonucleotides together with their melting points are given in table 1 trough 4.

Table 1

Melting points of oligonucleotides with a single anhydrohexitol nucleoside (A^*, T^*) incorporated (measured at 0.1 M NaCl-concentration) in the middle of an A_{13}/T_{13} duplex.

$d(T)_6Xd(T)_6$ $d(A)_6Yd(A)_6$				
Y\X	G	C	A	T
A	20.0	17.9	18.5	34.0
A*	20.2	17.1	17.7	32.1
X\Y	G	C	T	A
T	21.0	20.7	21.3	34.0
T*	15.1	15.2	18.3	28.7

From Table 1 it is clear that incorporation of 1,5-anhydro-2-(adenine-9-yl)-2,3-dideoxy-D-arabinohexitol into an oligodeoxyadenylate gives nearly identical helix-coil transitions as insertion of a natural 2'-deoxyadenosine. It should be mentioned, however, that one mismatch in a oligodeoxyadenylate/oligothymidine duplex has a large effect on duplex stability. On the contrary, substitution of thymidine by 1,5-anhydro-2,3-dideoxy-2-(thymin-1-yl)-D-arabinohexitol into an oligothymidylate gives a substantial decrease in melting temperature. In contrast to previous observations of our laboratory with 2,4-dideoxy-ß-D-erythro-hexopyranosyl nucleosides where an A*.G [A*:9-2,4-dideoxy-ß-D-erythrohexopyranosyl)adenine] mismatch gives more stable hybridization than an A*.T [A*:9-2,4-dideoxy-ß-D-erythrohexopyranosyl)adenine] base pairing (11) there is no alteration in base pairing specificity with the 1,5-anhydrohexitol nucleosides when using oligodeoxyadenylate/oligothymidine duplex as model.

Table 2
Melting temperature of completely modified oligonucleotides and of oligonucleotides modified at both ends, determined at 0.1 M NaCl.

	Tm(°C)	Hypochrommicity
equimol.mixt.with$(dA)_{13}$(14)		
$(dT)_6T^*(dT)_6$(8)	27.8	33%
$(T^*)_2(dT)_9(T^*)_2$(9)	27.6	32%
$(T^*)_{13}$(10)	45.4(1)	49%
equimol.mixt.with$(dT)_{13}$(15)		
$(dA)_6A^*(dA)_6$(4)	31.8	31%
$(A^*)_2(dA)_9(A^*)_2$(12)	30.3	33%
$(A^*)_{13}$(13)	21.0	49%
$(T^*)_{13}:(A^*)_{13}$(10:13)	76.3	ND
$(dT)_{13}:(dA)_{13}$(15:14)	34.0	35%

(1) measured at 284 nm

Single stranded oligoA* and oligoT* both show an ordered structure but, in contrast to the results at high salt concentration, (results not shown) polyT* does not show the same tendency for homoduplex formation. This is demonstrated by the more or less linear increase of the UV absorption with temperature, both for oligoA* and oligoT*. An equimolar mixture of oligoT* and oligodeoxyadenylate shows a melting temperature of 45°C with a hypochromicity of 49% when measured at 284 nm. It is known that, by changing salt concentration, structural transition occurs in DNA and this is here clearly the case. The oligoT*: oligodeoxyadenylate association is favored at lower salt concentration while the formation of oligoT* homoduplexes is favored at high salt concentrations. The thermal behavior of the complex at 260 nm, however, indicates that the oligoT*:oligodeoxy-adenylate association is not a classical helix-coil transition. At 260 nm, the hypochromicity first decreases, showing a minimum at 46°C (the melting point observed at 484 nm) and then increases. Fully modified mixed sequences (two hexamers and a dodecamer) containing the adenine (A*) and guanine (G*) nucleoside analogues have been evaluated likewise.

Table 3
Melting temperatures of fully modified hexamers

Sequence (equimol.mixt.with complement)		Tm(°C)
(16)	6'-A*G*G*A*G*A*	31.2
(17)	5'-AGGAGA	10.0
(18)	6'-G*A*G*A*G*A*	14.7
(19)	5'-GAGAGA	9.5

determined at 1M NaCl, 20 mM KH_2PO_4 pH 7.5, EDTA 0.1 mM

Duplexes were formed with the complementary sequences
5'-TCTCCT(20) for 16 and 17, and
5'-TCTCTC(21) for 18 and 19 respectively.

Although for some of these sequences melting points could be determined for the hexamers, thermal denaturation of these oligonucleotides was studied in 1 M NaCl (containing 20 mM K_2HPO_4 pH 7.5 and 0.1 mM EDTA). The most important phenomenon is the clear formation of a duplex between the pyranose-like oligonucleotides and their natural counterparts. Moreover, these modified duplexes are more stable than the control duplexes consisting of exclusively Watson-Crick base pairs.

Striking however is the large difference in melting temperature for sequences 16 (Tm = 31.2°C) and 17 (Tm = 14.7°C) with their antiparallel complementary oligonucleotides. Where both modified oligo's contain 3 G^*'s and 3 A^*'s differing only in their sequence order, the melting temperature for 16 doubles the one for 18. This sequence dependent effect is only marginally reflected by the control oligonucleotides 17 and 19.

Table 4
Melting temperatures of fully modified dodecamers containing A^* and G^*

Sequence (equimol.mixt.with complement)		Tm with 24(°C)
(22)	6'-A*G*G* G*A*G* A*G*G* A*G*A*	64.8
(23)	5'-AGG GAG AGG AGA	49.0

determined at 0.1 M NaCl

(24) 5'-TCT CCT CTC CCT

Looking at the dodecamers one notices again an increased stability of the fully modified oligonucleotide compared to its control sequence 23 with an increase in melting temperature of 16°C, when evaluating both sequences with their complementary antiparallel sequence 24.

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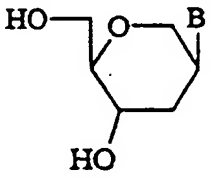
CLAIMS

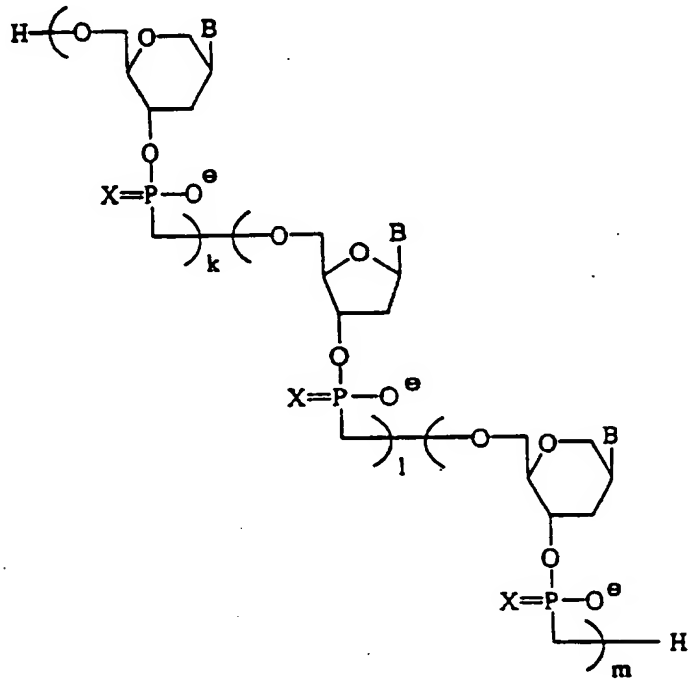
1. Oligomers consisting completely or partially of 1,5-anhydrohexitol nucleoside analogues represented by the general formula I



(I)

wherein B is a heterocyclic ring which is derived from a pyrimidine or purine base.

2. Oligomers as claimed in claim 1, **characterized** by the general formula II



(II)

wherein B is a heterocyclic ring which is derived from a pyrimidine or purine base, and

wherein k, l, and m each are integers from 0 to 15, provided k and m are at least one; but if k > 1, then m may be 0; and if m > 1, k may be 0; and,

wherein X represents oxygen or sulfur, and salts thereof.

3. Oligomers as claimed in claim 1 or 2, **characterized** in that the heterocyclic ring is selected from the group consisting of cytosine, 5-methylcytosine, uracil and thymine, or deaza derivatives thereof.

4. Oligomers as claimed in claim 1 or 2, **characterized** in that the heterocyclic ring is selected from the group consisting of adenine, guanine, 2,6-diaminopurine, hypoxanthine and xanthine, or deaza derivatives thereof.

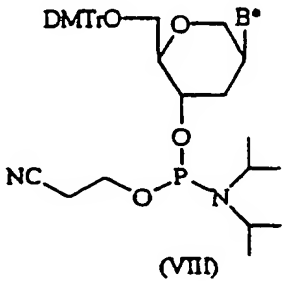
5. Oligomers as claimed in any one of the preceding claims, wherein the compound of formula I has the (D)-configuration and the substituents are located in the arabino-configuration.

6. Oligomers as claimed in any one of the claims 1-5 for use in antisense techniques.

7. Oligomers for the use as claimed in claim 6, **characterized** in that the antisense techniques comprise diagnosis, hybridization, isolation of nucleic acids, site-directed DNA modification and therapy.

8. Method of preparing oligomers of formula II, comprising coupling a suitable amount of monomers of formula I.

9. Phosphoramidites of the general formula VIII



(VIII)

wherein B^* is a protected base, for use in the preparation of oligomers of claim 1.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 95/03248

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H21/00 C07F9/6561 C07F9/6558 A61K31/70 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07H C07F A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,93 25565 (STICHTING REGA) 23 December 1993 cited in the application see claims ---	1-9
Y	US,A,5 314 893 (BRISTOL-MYERS SQUIBB CO.) 24 May 1994 see column 1 - column 3 --- -/--	1-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
19 December 1995	29. 12. 95

Name and mailing address of the ISA	Authorized officer
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International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NUCLEIC ACIDS RESEARCH, vol. 20, no. 18, 1992 OXFORD GB, pages 4711-4716, AUGUSTYNS K. ET AL. 'INCORPORATION OF HEXOSE NUCLEOSIDE ANALOGUES INTO OLIGONUCLEOTIDES: SYNTHESIS AND BASE-PAIRING PROPERTIES AND ENZYMATIC STABILITY' cited in the application see the whole document ---	1-9
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 95/03248

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		CA-A-	2111549	26-07-94
		EP-A-	0608809	03-08-94
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